RESULTS
&
DISCUSSION
Figure 1.1 Activation of resting B cells.
Purified B cells were either left untreated (black line) or treated with 10μg/ml anti-IgD (red line). The resultant effect on the various cell surface markers is depicted here.
Activation of resting B lymphocytes with anti-IgD antibodies.

Encounter of a T dependent antigen with the specific B cell surface receptor first occurs outside the follicles in T cell rich area. This leads to activation of resting B cells. Antigen activated B cells recruit several signaling pathways and undergo phenotypic changes, the most typical of which is a decrease in surface IgD levels. A variety of cell surface molecules are also upregulated. These include the costimulatory molecule CD86, the MHC class II, and the activation marker CD69 (Mond et al., 1981; Noelle et al., 1986; George and Claffin, 1992; Lenschow et al., 1994). Activated B cells subsequently adopt either of two distinct pathways of differentiation. They can either form foci of antibody secreting cells, or, enter into the splenic follicles to seed GCs where they differentiate into plasma or memory B cells (Nossal, 1994; Manser et al., 1998).

As discussed earlier, not all antigen activated B cells migrate to the follicles. Rather, they undergo a stringent selection process in which only the high affinity B cells are recruited to seed the GCs (Agarwal et al., 1998). Activated B cells within the GCs are characterized by the expression of several additional cell surface markers such as the receptor for peanut agglutinin (PNA-R), CD24, CD95, and the activation marker GL7 (Rose et al., 1980; Hardy et al., 1984; Lalor et al., 1992; Han et al., 1997; Kimota et al., 1997; Lahvis and Cerny, 1997; Koni et al., 1999). Thus, the affinity of BCR for antigen appears to play an important role in defining the phenotypic as well as the functional status of antigen stimulated resting B cells.

To examine pathways mediating phenotypic alterations in resting B lymphocytes, the F(\(\text{ab}\))\(_2\) fragment of an anti-IgD mAb was employed as the surrogate antigen (hereafter referred to as anti-IgD). Purified resting B cells were co-cultured with anti-IgD, following which cell surface modulation of select proteins that typify activated and GC B cells was monitored. The results thus obtained are shown in Figure 1.1. As expected, sIgD levels were downregulated whereas surface densities of CD86 and MHC class II molecules were markedly enhanced (Fig. 1.1). However, additional markers that characterize the GC phenotype of activated B cells were also found to be upregulated. These included the receptor for peanut agglutinin (PNA-R), the activation marker GL7, and the heat stable antigen CD24. Finally, apart from these two groups of markers
surface expression of the costimulatory molecule, CD80, was also found to be enhanced upon stimulation of resting B cells with anti-IgD. Interestingly, all of the responses observed in Figure 1.1 were completely inhibited when stimulation was performed in the presence of the broad spectrum protein tyrosine kinase (PTK) inhibitor, genistein (Akiyama and Ogawa, 1991). These latter results imply the dependence of the effects observed in Figure 1.1 on tyrosine phosphorylation events initiated upon BCR ligation.

To further characterize the induction of the markers seen in Figure 1.1, corresponding mRNA levels were also analyzed. However, since the gene coding for GL7 is presently unknown, while the PNA- binding activity derives from the DGalβ-1-3DGalNAc residues on surface glycoproteins that are presumably unmasked in GC B cells, transcripts for only CD80, CD24, CD86, and I-A could be examined. As shown in Figure 1.2, a Northern blot analysis revealed that stimulation of B cells with anti-IgD also resulted in increased levels of mRNA for all the four proteins examined. Thus, the increased surface densities of these molecules, upon stimulation with anti-IgD, probably derived from the observed increase in their corresponding mRNAs. However, whether the latter reflects enhanced message stabilization, or, increased transcriptional activation of the corresponding genes is presently unknown.

![Figure 1.2 Northern Blot analysis for cell surface markers.](image)

RNA was extracted from both unstimulated cells (U) and cells stimulated (S) with anti-IgD. Northern blots were analyzed using cDNA probes specific for I-A, CD86, CD80 and CD24 (panel A), and for β-actin (panel B). For CD86, and I-A cells were stimulated for 24 hrs, whereas for CD24, and CD80 stimulation period was 48 hrs.

Resting B lymphocytes stimulated with anti-IgD, which exhibit the activated and the GC phenotype, were also analyzed for their GC seeding potency by using a modified
adoptive transfer protocol that we have standardized earlier. In this procedure, resting B cells from BALB/c IgHa mice were first treated with anti-IgD for 48 hrs and then loaded with a synthetic peptide CT3 (sequence: DIEKKIAKMEKASSVFNVVNS) representing a promiscuous T cell epitope derived from the circumsporozoite protein of malaria parasite, *Plasmodium falciparum* (Materials and Methods). This culture was continued for an additional 12 hr period. Anti-IgD stimulated, peptide CT3 loaded cells were then transferred into BALB/c IgHb mice that had been primed, four days earlier, with peptide CT3 (Materials and Methods). At eight days after transfer, spleens were removed and sections were stained for IgHa allele specific B cells, which were also positive for PNA-R. Between 80-90 GCs per 10 sections were observed in the spleens of mice where anti-IgD stimulated cells were transferred (Fig. 1.3). In separate experiment it was ascertained that CT3 loading by itself had no further influence on the cell surface phenotype of anti-IgD stimulated cells.

The requirement for CT3 loading and priming was found to be obligatory as stimulated cells loaded with an irrelevant peptide prior to transfer into CT3 primed mice did not develop into germinal centers. Similarly, stimulated cells when loaded with CT3 but transferred either into unprimed mice, or mice primed with an irrelevant peptide, failed to form germinal centers. Virtually no GCs could be observed when unstimulated cells were loaded with CT3 and transferred into CT3 primed mice (Fig. 1.3). Thus anti-IgD stimulation of resting B cells not only alters the phenotypic status of B cells, it also induces the functional capability of B cells to populate GC. However, the latter occurs strictly in T-dependent manner.

We also monitored the kinetics of GC formation obtained with the above procedure. Although small clusters of allele-specific and PNA+ cells could be detected as early as three days after adoptive transfer, distinct and fully formed GCs could only be detected at day 5 (Fig. 1.4). Their number increased with time and reached at maximal levels by day 8. Following this, however, the numbers declined such that virtually no GCs were detectable by day 15 (Fig. 1.4).
Anti-IgD treatment gives B cells the potential to seed germinal center. Purified resting B cells from BALB/c IgH\textsuperscript{a} mice were either left unstimulated or stimulated with 0.1\,\mu\text{g}/ml of anti-IgD. Cells were loaded with peptide CT3 and transferred into CT3 primed BALB/c IgH\textsuperscript{a} mice. Representative spleen sections, doubly stained for PNA positive and IgH\textsuperscript{b} allele specific GCs are shown in panel a (100 x magnification) and b. Shown in panel d is a spleen section from recipient where unstimulated cells were transferred. Spleen section from unprimed mice is also shown (panel c).
Figure 1.5 Effect of EGTA on the surface induction of markers.
Cells were either left untreated (thin line) treated with anti-IgD either in the presence (dashed line, or absence (thick line)of 3mM EGTA. Resultant effects on the surface upregulation of markers is shown here.
Figure 1.4 Kinetics of Germinal Center formation.

Anti-IgD treated (Blue curve) or untreated (Red curve) cells were transferred in the CT3 primed BALB/cIgH\* mice. Spleens were removed from the mice at the indicated times and sections were processed for the PNA positive and allele specific staining. Number of GCs averaged over ten sections are represented here.

Second messenger mediators of BCR-dependent pathways.

Upon BCR crosslinking diverse signaling cascades are initiated which results in the generation of multiple second messengers. As discussed earlier, it is this recruitment of second messengers that eventually leads to transcription factor activation and gene expression. Thus, surface expression of the activation and GC markers shown in Figure 1.1 is also probably mediated by specific second messengers recruited upon anti-IgD stimulation of resting B cells. To identify at least some of the important second messengers that might drive the activation of resting B cells, the consequences of addition of a variety of pharmacological inhibitors, was first examined. For this, cells were stimulated with anti-IgD in the presence of EGTA, Calphostin C (Cal C), or H-89. EGTA is a known chelator of extracellular calcium ions (Ca^{2+}) and thus blocks the Ca^{2+}-influx from the extracellular milieu (Dolmetsch et al., 1997, 1998). Calphostin C, specifically inhibits protein kinase C (PKC) activation (Chao et al., 1992), whereas H89 is an inhibitor of the cyclic AMP-dependent Ser-Thr kinase, protein kinase A (PKA) (Rooij et al., 1998). Results from such experiments revealed that stimulation in the
Figure 1.6 Effect of Calphostin C in anti-IgD mediated induction of cell surface markers. Purified B cells were either left unstimulated (thin line) or stimulated with anti-IgD (10μg/ml) either in the presence (dashed line) or absence (thick line) of 0.1μM Calphostin C. The resultant effects on the surface markers are shown as FACS profiles.
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Presence of EGTA completely inhibited upregulation of all the activation and GC markers as shown in Figures 1.5 and 1.8. This was also true of calphostin C where the anti-IgD dependent upregulation of surface markers was again inhibited (fig. 1.6 and 1.8). H89 also produced inhibitory effects, although the extent of inhibition varied from partial to complete depending upon the marker examined (fig 1.7 and 1.8). Similar results were also obtained when alternate inhibitors of Ca\(^{2+}\) mobilization (TMB-8), PKC activation (staurosporine), or PKA activation (H-9) were employed. These results, therefore, implicate that BCR-dependent induction of the cell surface molecules involves at least three distinct intracellular second messengers - Ca\(^{2+}\), PKC, and cAMP.

![Graph showing relative MFI for CD86, PNA-R, I-A, GL7, CD24, and CD80](image)

Figure 1.8 Second messenger mediators of B cell activation.

Purified resting B cells were either left untreated or treated with anti-IgD (10μg/ml) either in the absence or presence of either EGTA (3mM), Calphostin C (0.1μM), or H89 (100nM). The resultant levels of individual surface markers are shown here.

To confirm the above findings, the direct effects of these three second messengers were also examined, under conditions where they were mobilized independently. Influx of Ca\(^{2+}\) from the extracellular milieu can readily be obtained by the use of ionophores such as Ionomycin (Dolmetsch et al., 1997), whereas intracellular cAMP concentrations can be enhanced by employing its acylated analog, dibutryl-cAMP (db-cAMP) (Nabavi et al., 1992). In vivo activation of the Ser/Thr kinase, PKC, has been commonly achieved.
Figure 1.7 Effect on H-89 on surface induction of phenotypic markers.

Purified resting B cells were stimulated with anti-IgD (10μg/ml) either in the presence (dashed line) or absence of 100nM H-89 (thick line). Resultant effects on individual markers is shwon as FACS profile. For comparison profiles for unstimulated cells (thin line) are also shown.
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by the phorbol ester, phorbol-12-myristate-13-acetate (PMA) (Xie and Rothstein, 1995). Purified resting B cells were independently stimulated with saturating concentrations of each of these agents, and the consequent effects on the cell surface phenotype were monitored. As shown in Figure 1.9, treatment of cells with either ionomycin or PMA proved to be as effective as the anti-IgD stimulus, where all the cell surface molecules examined were upregulated. Indeed, the extent of induction observed was comparable with that obtained upon stimulation of cells with anti-IgD (Fig. 1.9).

Figure 1.9 Effect of Ionomycin, PMA and db cAMP on the surface induction of markers.

Cells were stimulated with either 10μg/ml of anti-IgD, 100μM Ionomycin, 10nM PMA or 600μM db cAMP. The resultant levels of individual surface markers are represented in terms of modal fluorescence intensity.

In contrast to the effects of ionomycin and PMA, the potency of db-cAMP was markedly reduced, yielding enhancements that ranged from marginal to moderately significant depending on the marker (Fig 1.9). The results in Figure 1.9 could be further confirmed by the fact that alternate stimulators of either Ca²⁺ (with the ionophore A23187), PKC activation (with PDBu), or, cAMP (with Forskolin) yielded similar effects. Importantly, the stimulatory influence of ionomycin could be inhibited by the
addition of EGTA, but not by either CalC or H89. On the other hand, activation of PMA was completely sensitive to CalC, partially sensitive to EGTA (40-60% inhibition), but insensitive to the addition of H89. Finally, the sensitivity of db-cAMP-mediated effects was restricted to H89, with no inhibition in the presence of either EGTA or CalC. These findings, therefore, suggests that at least partially non-overlapping downstream pathways regulate the effects of the above three mediators.

**Recruitment thresholds of second messengers mediating BCR signaling.**

Although the expression of various cell surface markers could be artificially induced by increasing either intracellular Ca^{2+}, PKC activity, or cAMP levels (Fig. 1.9), we recognized that such findings were only meaningful when examined in a physiologically relevant context. This was particularly true given the apparent redundancy of pathways mediating induction of the molecules monitored in Figure 1.9. Thus, the extent to which these mediators were recruited, upon stimulation of resting B cells, was determined. The actual concentration of Ca^{2+} mobilized upon anti-IgD triggering in resting B cells was first measured. Consistent with prior findings (Cambier and Ransom, 1997), a biphasic profile for Ca^{2+} recruitment was observed (Fig. 1.10a). Upon addition of anti-IgD, a rapid, but transient increase was immediately detected, which declined rapidly to stabilize at concentrations that were still significantly above the base line (Fig. 1.10a). It has been previously shown (Cambier and Ransom, 1997) that the first, rapid phase results from the IP3-mediated release of Ca^{2+} from the intracellular stores. On the other hand, the second phase of low but sustained elevation of Ca^{2+} represents the capacitative influx mediated by the calcium-release activated channels (CRAC) (Cambier and Ransom, 1997). As expected anti-IgD-dependent Ca^{2+} mobilization could be completely inhibited by TMB-8, an inhibitor of IP3-mediated release from intracellular stores (Chao et al., 1992) (Fig. 1.10a). In contrast to this, the effect of EGTA addition was more selective where only the capacitative influx phase of Ca^{2+} recruitment was inhibited, with virtually no effect on release from the intracellular pool (Fig. 1.10a). As shown in figure 1.5, EGTA, which blocks the capacitative influx completely inhibited the anti-IgD mediated induction of markers. These results suggest
that the second phase of \( \text{Ca}^{2+} \) mobilization – the capacitative influx- plays an obligatory role during the anti-IgD mediated phenotypic modifications of resting B cells. We determined the ionomycin concentration that would replicate the capacitative influx phase of \( \text{Ca}^{2+} \) upon stimulation of resting B cells with anti-IgD. For this, cells were treated with varying concentrations of ionomycin, and the resultant \( \text{Ca}^{2+} \) influx was measured. The concentration of \( \text{Ca}^{2+} \) recruited during the capacitative influx phase of anti-IgD dependent stimulation was readily achieved by as low as 10nM concentration of ionomycin (Fig. 1.10b).\(^3\)

**Results 1.10 Anti-IgD dependent \( \text{Ca}^{2+} \) mobilization.**

*In panel a, FLUO-3-AM loaded B cells were stimulated with 10 \( \mu \text{g/ml} \) of anti-IgD either in the absence (profile 1) or presence of either EGTA (profile 2), or TMB-8 (profile 3). The resultant recruitment of \( \text{Ca}^{2+} \) was monitored by flow cytometry. In panel b, these cells were treated with indicated concentrations of ionomycin, and the resultant influx of \( \text{Ca}^{2+} \) averaged over a 10 min period is plotted. The line intersecting the plot indicates the mean \( \text{Ca}^{2+} \) concentration obtained during the capacitative influx phase of the anti-IgD stimulated response.*

Similarly, the extent of PKC activated upon anti-IgD stimulation of the cells was also examined. This was measured as translocation of PKC activity from the cytosolic to the membrane fraction, and the results are presented in Figure 1.11a. As expected anti-IgD dependent PKC activation was completely inhibited by Calphostin C. Activation of at least conventional isoforms of PKC depends upon DAG and \( \text{Ca}^{2+} \).
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(Leitges et al., 1996). To analyze the $\text{Ca}^{2+}$ dependency, PKC activity was measured in the anti-IgD stimulated cells pretreated with EGTA or TMB-8. As shown in figure 1.11a, EGTA only partially inhibited the anti-IgD-dependent PKC activation, whereas the addition of TMB-8 completely blocked the PKC activation. This implies that the PKC activity is greatly dependent upon the $\text{Ca}^{2+}$ released from the intracellular stores. Further, by using varying concentrations of PMA it was revealed that a 0.2 nM concentration of PMA reproduced the effects of anti-IgD on PKC activation (Fig. 1.11b).

![Graph showing PKC activity](image)

**Figure 1.11 Anti-IgD dependent PKC activation.**

Panel a depicts the ratio of membrane (m) to cytosolic (c) activity of PKC in either unstimulated cells (cells), or cells stimulated with anti-IgD (with 10 µg/ml) either in the absence or presence of Calphostin C (Cal C), EGTA or TMB-8 (TMB). Panel b shows the effects of treatment with indicated concentrations of PMA on PKC translocation, and the line intersecting the graph represents the extent of PKC activation obtained upon anti-IgD stimulation.

As discussed earlier, the anti-IgD mediated induction of markers was also inhibited by H-89. This implies that the signaling pathways recruited upon anti-IgD stimulation were also PKA dependent. Activation of PKA normally requires induction of cAMP. Interestingly, recruitment of intra-cellular cAMP not been reported in BCR mediated signaling (Xie and Rothstein, 1995). Thus, the effects of anti-IgD on intracellular cAMP (cAMP$_i$) levels in murine B cells were also examined. As shown in Figure 1.12a, stimulation with anti-IgD resulted in a near ten-fold increase in cAMP$_i$ concentrations. The magnitude of this enhancement was comparable to that obtained.
upon I-A crosslinking, which in murine resting B cells is known to induce cAMP. Importantly, the enhancement in cAMP₁ levels was completely unaffected by the inclusion of either Cal C, EGTA or TMB-8 in the cultures (Fig. 1.12a). This suggests that accumulation of cAMP₁, in response to anti-IgD stimulation, occurred by a mechanism that was independent of Ca²⁺ signaling. Finally, as shown in Figure 1.12b, the effect of anti-IgD on cAMP₁ levels could be duplicated by the extraneous addition of 100 μM of its analog, db-cAMP.

**Figure 1.12 Anti-IgD dependent induction of cAMP₁ in resting B cells.**

Cells were treated with anti-IgD (10 μg/ml) in the absence or presence of various inhibitors indicated and the resultant effects on the cAMP₁ response are represented in panel a. Panel b shows the extent of cAMP₁ accumulation as a result of treatment with varying concentrations of db-cAMP. The line intersecting the graph indicates the cAMP₁ concentrations obtained upon stimulation with anti-IgD.

**Crosstalk between intracellular mediators of sIgD-signaling.**

After establishing the extent of anti-IgD mediated recruitment of the above intracellular mediators, their effects at these particular concentrations were next examined. For this, resting B cells were individually stimulated with either 10 nM ionomycin, 0.2 nM PMA, 100 μM db-cAMP, or, varying combinations of these three agents. With the exception of CD86, surface levels of all the other molecules were relatively unaltered when B cells were treated with each of the above agents separately (Fig. 1.13) Surface densities of
CD86 were, however, enhanced by about four-fold in the presence of 0.2 nM PMA (Fig. 1.13). These results therefore suggest that although the upregulation of surface markers was dependent upon the recruitment of second messengers, the individual levels at which these intracellular mediators were recruited by sIgD cross-linking were insufficient to independently ensure upregulation of the activation/GC markers studied. The only exception to this was CD86, also implying that distinctions exist in induction thresholds for these molecules.

In contrast to their low individual potency, combinations of these agents – however – proved significantly more effective, eventually yielding maximal upregulation of all the markers measured. Thus, for example, use of PMA in combination with either ionomycin or db-cAMP resulted in a significant enhancement in surface densities of CD86, I-A and GL7 (Fig. 1.13). Indeed, in the case of I-A, either of these two combinations was alone sufficient to reproduce the effects of anti-IgD stimulation (Fig. 1.13). This ability to co-opt, in conjunction with PKC activation, either Ca\textsuperscript{2+} or cAMP-signaling pathways with equal facility for MHC class II induction may well identify an inherent versatility of resting B cells. This, in turn, would likely ensure ready acquisition of productive antigen presentation capability by resting B cells.

In contrast to the selective effects of dual stimulation, treatment of cells with a combination of all three agents – ionomycin, PMA and db-cAMP – resulted in upregulation of all the markers examined. Indeed, the extent of induction was comparable to that obtained upon stimulation with anti-IgD in all cases (Fig. 1.13). The results in Figure 1.13, therefore, highlight two important principles that appear to regulate activation of resting B cells. First, they further confirm the existence of distinct thresholds, leading to a hierarchy of signaling requirements for induction of the independent surface molecules examined. Thus, while suboptimal PKC stimulation sufficed to induce CD86 expression, upregulation of the MHC class II molecule required the combined effects of suboptimally stimulated PKC- and either Ca\textsuperscript{2+} or cAMP-dependent pathways. As opposed to this, optimal enhancement of the remaining molecules examined depended upon contributions from signaling pathways deriving from all the three, suboptimally recruited, second messengers (Fig. 1.13).
An important consequence of the non-identical induction requirements noted above is that they also translate into distinct signaling thresholds that define the extent to which phenotypic modification of resting B cells is achieved. Thus, while downstream products of the BCR-dependent phosphoinositol signaling pathway, Ca^{2+} and PKC, were sufficient to confer the activated phenotype (i.e. I-A^{hi}, CD86^{hi}), acquisition of at least a partial GC phenotype required additional cooperation from cAMP-dependent signaling (Fig. 1.13). In a similar vein, a hierarchy in signaling requirements for different markers of human GC B cells was also noted by Galibert et al (1996).

**Figure 1.13 Activation of B cells depends upon the crosstalk between intracellular mediators.**

*Cells were either left unstimulated (cells), or stimulated either with 10nM ionomycin (In), 0.2nM PMA (P), 100μM db-cAMP (D), or the indicated combinations of these three agents.*
comparative purposes, a parallel set of cells stimulated with 10 µg/ml of anti-IgD (aIgD) is also shown. The resultant effects on the surface markers are depicted in terms of modal fluorescence intensity.

A second important principle also becomes evident from a comparison of the data in Figures 1.9 and 1.13. Thus, while at least some of the downstream mediators were fully capable of conferring both the activated and GC phenotype to resting B cells, they were recruited only at suboptimal levels upon BCR cross-linking. This, in turn, necessitated crosstalk between these intracellular mediators where the extent of this interaction defined the spectrum of functionally relevant molecules that were expressed.

The effectiveness of crosstalk between these intermediates, in terms of regulating activating thresholds, is especially highlighted by the data in Figure 1.14. This figure depicts the effects of treating cells with varying doses of PMA on a representative set of molecules. Further, the effects of inclusion of either ionomycin (10 nM) alone, or ionomycin (10 nM) plus db-cAMP (100 µM), on the dose response to PMA are also shown here. At the simplest level, these results further underscore the existence of independent activation thresholds for the markers examined. Thus, while peak expression of CD86 was obtained in the presence of 0.2 nM PMA, that for both I-A and GL7 required PMA concentrations of at least 10 nM (Fig. 1.14). In addition, consistent with the low PKC-threshold requirement, no further effect from the addition of either ionomycin alone, or, ionomycin plus db-cAMP was noted for CD86 levels (Fig. 1.14). In contrast with this, PMA dose requirements for MHC class-II and GL7 upregulation were shifted to significantly lower concentrations in the added presence of ionomycin (Fig. 1.14). This effect was further accentuated when the combination of ionomycin plus db-cAMP was employed instead of ionomycin alone (Fig. 1.14). Importantly, the magnitude of enhancement of I-A and GL7 was also affected when PMA stimulation was supplemented either with ionomycin alone, or with the combination of ionomycin plus db-cAMP (Fig. 1.14). Thus the maximal surface densities of GL7 obtained with PMA alone was increased in the presence of PMA plus ionomycin. This was further amplified when db-cAMP was also included (Fig. 1.14). The corresponding results obtained for I-A, however, were somewhat surprising. While the addition of ionomycin and db-cAMP reduced the PMA concentration requirements for MHC class-II induction, markedly
higher peak levels were – nonetheless – obtained when only PMA and ionomycin were present (Fig. 1.14). The significance of these latter observations is not clear at the present time. Finally, consistent with the observations in Figure 1.13, induction of I-A was less dependent upon the presence of db-cAMP than was GL7 (Fig. 1.14).

**Figure 1.14 Crosstalk between the second messengers.**

Cells with treated with varying concentrations of PMA either in the absence (blue curves) or presence of 10 nM ionomycin alone (red curves), or, ionomycin plus 100 μM db-cAMP (green curves). The resultant effects on GL7, I-A, and CD86 is depicted in terms of mean fluorescence intensity.

The results in Figure 1.14 unambiguously demonstrate that cooperativity between BCR-dependent second messenger pathways influences, in both qualitative and quantitative terms, phenotypic responses to an antigenic stimulus. Importantly, they also provide a mechanistic insight by revealing how distinct threshold requirements for a
given signaling module can be overcome by contributions from the other subsidiary pathways activated by BCR cross-linking. Thus, while the PMA dose requirement for CD86 upregulation differed from that for I-A and GL7 this, however, was normalized when both Ca\textsuperscript{2+} and cAMP\textsubscript{i} were also simultaneously mobilized.

**Membrane distribution of sIgD**

As shown in Figure 1.10-1.12, anti-IgD dependent stimulation of resting B cells resulted in the recruitment of the phosphoinositol and the cAMP pathways. Thus it was of interest to examine how these two independent signaling pathways are generated from the BCR. These results, however, also raised the possibility that these two pathways could be spatially segregated. Recently it is becoming clear that receptor mediated signaling in lymphocytes occurs in discrete, and specialized domains on the cell membrane termed as lipid rafts. As discussed in literature review, lipid rafts represent cholesterol and glycosphingolipid-enriched membrane domains and are detergent insoluble in nature (Horejsi et al., 1999; Langlet et al., 2000; Cherukuri et al., 2001). Lipid rafts function as platforms for various biological processes such as signaling and membrane trafficking (Simons and Ikonen, 1997). Signalling for various immune receptors including the BCR, the TCR, the FcεRI, and the MHC class II molecule is known to be initiated in the lipid rafts (Anderson et al., 2000; Langlet et al., 2000; Cherukuri et al., 2001). In both pre-B and mature B cells, the antigen receptor has been shown to be predominantly present in the detergent soluble membrane fraction in unstimulated cells. Cross-linking of these receptors, however, induces translocation of a significant fraction of these receptors into lipid rafts with the subsequent formation of multi-protein signaling complexes (Cheng et al., 1999; Guo et al., 2000; Cheng et al., 2001). Recent results suggest that BCR translocation into rafts requires the integrity of the transmembrane domain, and probably precedes activation of the BCR-associated Src family of PTKs (Cheng et al., 2001; Dykstra et al., 2001). In contrast to mature B cells, BCR translocation to rafts was not observed in either immature or tolerant cells; suggesting that such distinctions may account for the diversity in physiological responses to antigens by these cell types (Sproul et al., 2000; Weintraub et al., 2000; Chung et al., 2001).
We examined the membrane distribution of slgD in murine resting B cells. For this, plasma membrane domains were isolated from unstimulated and anti-IgD stimulated cells based on their insolubility in Triton X-100 at low temperatures, and low buoyant density in sucrose gradients (Cheng et al., 1999; Guo et al., 2000). Aliquots of each fraction were then resolved by gel electrophoresis and slgD was detected with anti-IgD antibodies by western blot analysis.

As expected the majority of slgD receptors in unstimulated resting B cells were present outside the lipid rafts in the soluble fraction of the membrane (Fig. 1.15). Only a small component could be detected in the detergent-insoluble fraction (Fig. 1.15). Further, cross-linking with anti-IgD induced translocation of a significant proportion of slgD receptors into the raft fraction (Fig. 1.15). Detergent-insoluble membranes are characterized by the enrichment of glycosphingolipids and lipid modified proteins. The GM1 ganglioside represents a well characterized marker of lipid rafts (Xavier, et al., 1998). Cholera Toxin B subunit (CTB) binds to GM1 ganglioside, therefore, specifically recognize the raft region of the plasma membrane. Lipid rafts exclude transmembrane proteins. Figure 1.15 shows that CTB binding was highly specific to detergent insoluble fractions and was detected in very low level in detergent soluble fractions. In contrast, the transmembrane protein CD45R (or B220) was excluded from the detergent insoluble fractions.

![Figure 1.15 Membrane distribution of slgD receptors.](image)

*Resting cells were lysed in Triton X-100 and detergent insoluble (fractions 4-6) and soluble (fractions 10-12) fractions were separated. 30 μl sample was resolved on a 10% polyacrylamide*
gel and then transferred onto a nitrocellulose membrane. These were then immunoprobed for either GM1-ganglioside using biotinylated cholera toxin B subunit (CTB), or for either IgD (slgD), or CD45R (B220). The distribution of slgD receptors in unstimulated cells, and in cells stimulated with 10μg/ml of anti-IgD (5 min at 37º C) is shown in the panels identified as slgD (u) and slgD(s) respectively. The lowest panel, described as CTB (mcd), shows the effect of treatment of cells with MCD (10 mM) on GM1-gangliosides.

As discussed earlier integrity of rafts depends upon the presence of cholesterol. Thus, rafts can be disrupted by adding cholesterol extracting agents such as nystatin or methyl-b-cyclodextrin (MCD). Whereas nystatin perturbs raft domains by sequestering cholesterol in cell membrane (Bolard et al., 1986), MCD extracts cholesterol from plasma membrane by complexing into a hydrophobic pocket rendering it soluble in aqueous medium (Klein et al., 1995). As shown in figure 1.15, addition of MCD to the stimulated B cells resulted in the disruption of rafts. Further, the effects of both MCD and nystatin were found to be reversible, as removal of these agents from the culture resulted in a reconstitution of the raft domains when monitored in membrane fractions prepared one hour later.

Membrane compartmentalization of slgD signaling and the receptor subsets

The dependency of slgD-signaling on the integrity of lipid rafts was also examined. For this cells were treated with anti-IgD in the presence of 10mM MCD or 50μg/ml nystatin and resultant Ca\textsuperscript{2+} mobilization and cAMP induction was monitored. As shown in Figure 1.16a, stimulation of cells in the presence of either MCD or nystatin had no effect on the Ca\textsuperscript{2+}-response. These results are consistent with the earlier observations of Petrie et al., who also did not detect any effect of raft-disrupting agents on BCR-dependent Ca\textsuperscript{2+} mobilization in primary human B cells.

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Figure 1.16 Membrane compartmentalization of IgD signaling.

Purified cells were stimulated with anti-IgD (10μg/ml) either in the absence (profile 1 in panel a, cells in panel b) or presence of either 50 μg/ml nystatin (profile 2 in panel a) or 10 mM MCD (profile 3 in panel a). Following this, the effects on both Ca^{2+} (panel a) and cAMP (panel b) were monitored.

In contrast to the insensitivity of the Ca^{2+}-response, slgD-dependent recruitment of cAMP was, however, highly dependent on the integrity of raft micro-domains. The enhanced level of cAMP obtained in anti-IgD-treated cells was completely abrogated when stimulation was performed in the presence of either nystatin or MCD (Fig. 1.16b). Thus the Ca^{2+} and cAMP -signaling pathways that result from slgD cross linking, appear to emanate from non-identical compartments of the cell membrane.

Wienands and Reth have suggested that, in addition to association with CD79a/CD79b, IgD receptors can also be expressed on the surface by linkage to glycosyl-phosphatidylinositol (GPI) anchor (Wienands and Reth, 1992). This was demonstrated by transfection of slgD into CD79a/CD79b-deficient cells, although whether this occurs in normal resting B cells is presently unknown. Further, GPI-anchored proteins are known to normally localize within lipid rafts (McConville and Ferguson, 1993; Varma and Mayer, 1998; Friedrichsen and Kurzchalia, 1998). Therefore, the possibility of distinct isoforms of slgD driving two spatially distinct anti-IgD-dependent biochemical activities i.e. Ca^{2+} mobilization and cAMP induction, was analyzed. For this phosphatidylinositol-specific phospholipase C (PI-PLC), an enzyme
that specifically releases GPI-linked proteins from the cell surface, was employed. Wienands and Reth have demonstrated that the PI-PLC treatment completely released the GPI-linked slgD from the transfected cells (Wienands and Reth, 1992). The specificity of PI-PLC treatment was also ascertained in anti-IgD treated cells. The surface expression of the GPI-linked CD24 was completely abolished by PI-PLC treatment, whereas it had no effect on the transmembrane protein CD86.

To probe for the possible presence and membrane distribution of PI-PLC-sensitive forms of slgD, resting cells that were either untreated or treated with PI-PLC were first surface-biotinylated. Cells were lysed in TX-100 and subjected to sucrose density ultracentrifugation. Detergent soluble and insoluble fractions of the plasma membrane were pooled separately and incubated with FITC labeled anti-IgD. Following this, biotinylated proteins were adsorbed onto the streptavidin coated magnetic beads and the amount of fluorescence present in each fraction was monitored. Results thus obtained are represented in Figure 1.17a. Treatment with PI-PLC had no significant effect on the amount of slgD present in the detergent soluble fraction of unstimulated cells. On the other hand, the minority population present in the detergent insoluble fraction was reduced by a little over fifty per cent (Fig. 1.17b). In parallel experiments, treatment with PI-PLC did not alter surface I-A and B220 levels present in either the detergent soluble, or, detergent insoluble fractions of membranes from resting B cells. These results, therefore, strongly suggest that a significant proportion of the raft-associated slgD receptors, in unstimulated resting B, cells exist in a form that is sensitive to PI-PLC treatment. The quantitation of the levels of slgD present in both the compartments was also carried out. While the 95-96% of the total IgD was present in soluble fractions only a minor population (4-5%) was localized in rafts.

Effect of PI-PLC treatment on the second messenger recruitment following BCR stimulation was also examined. PI-PLC treatment had no effect on the Ca\(^{2+}\) mobilization in anti-IgD stimulated cells. On the other hand, the cAMP response in anti-IgD treated cells was completely abrogated in PI-PLC treated cells (Fig. 1.17b). Further, the cAMP derived from the crosslinking of MHC class-II, which is a transmembrane receptor remained unaffected by the PI-PLC treatment (Fig. 1.17b). These results imply that the Ca\(^{2+}\) response obtained upon anti-IgD stimulation of resting B cells is derived from the...
transmembrane form of receptor whereas it is the PI-PLC sensitive isoform of raft-associated sIgD that specifically generates the cAMP₁ response.

![Graph showing distinct isoforms of surface IgD receptor.](image)

**Figure 1.17 Distinct isoforms of surface IgD receptor.**

In panel a, PI-PLC (1 unit/ml) treated or untreated cell were first surface biotinylated with NHS-Sulpho-Biotin and detergent soluble (DS) and insoluble fractions (DIS) were separated. The presence of sIgD was determined by incubating with anti-IgD FITC. The values on Y-axis represent relative fluorescence units. Panel b represents the effect of PI-PLC treatment on the cAMP response where both PI-PLC untreated (aIgD) and PI-PLC treated (PLC+aIgD) cells were stimulated with anti-IgD (10μg/ml). As a control, an additional group was included where either untreated (aI-A) or PI-PLC treated (PLC+aI-A) cells were stimulated with 10μg/ml of an anti-I-A mAb. Panel B shows the resultant increase in cAMP₁ obtained in these groups.

**Identification of a GPI-linked subset of sIgD**

The GPI-anchor that links proteins to membrane phospholipid is a complex structure that is composed of ethanolamine, mannose, glucose and inositol residues. Further characterization was done in order to confirm that the PI-PLC sensitive subset of receptor observed in Figure 1.17a actually represents a GPI-linked isoform. For this, purified resting B cells were independently pulsed with radioactive analogues of either glucosamine, mannose, or a mixture of the fatty acids palmitic and myristic acid in the...
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presence of tunicamycin which is the known inhibitor of N-linked glycosylation (Material and Methods). Following this, detergent soluble and insoluble membrane fractions were separated, and incubated with anti-IgD antibody. The immunoprecipitated sIgD was then de-glycosylated as discussed in materials and methods, and extent of radioactivity incorporated into each fraction was then determined. As shown in Figure 1.18, although the majority of the sIgD receptor was associated with the detergent insoluble fraction, the amount of radioactivity incorporated was negligible in all the groups. In contrast to this, the minor population of sIgD, which was localized in the raft fraction co-purified with glucosamine, mannose, and also fatty acids (Fig. 1.18). As mentioned earlier, these three chemical entities are collectively present only in GPI-anchored proteins (Fig. 1.18). Further, the association of fatty acids with sIgD could be completely abolished by the PI-PLC treatment. No significant incorporation of radioactivity was detected in experiments where immunoprecipitations were done with either anti-mouse IgM, or non-specific rat IgG.

In a parallel experiment, detergent soluble and insoluble fractions were also isolated using alternate protocol. These radiolabelled cells were lysed in Triton-X114, subjected to phase separation at 37°C, and analyzed for the incorporation of radioactivity. Radioactivity was found to be associated with only the raft fraction with no detectable counts in the soluble fraction. These collective results thus provide additional evidence for the expression of a minority population of sIgD, which is constitutively localized in the lipid rafts, is mediated by a GPI-linkage.
Figure 1.18 Identification of GPI-linked sIgD.

Cells were pulsed with radiolabeled analogues of either glucosamine (GA), mannose (MN), or a mixture of the fatty acids palmitic and myristic acid (FA) in the presence of tunicamycin (as discussed in methods). Immunoprecipitates with anti-IgD antibodies from the detergent-soluble (DS) and detergent-insoluble (DIS) fractions of the cell membrane were then de-glycosylated, and analyzed for incorporated radioactivity. Immunoprecipitates from fatty acid labeled cells were also treated with PI-PLC prior to analysis (FA+PLC).

To further confirm that the data in Figure 1.18 identify a GPI-linkage, intracellular GPI-biosynthesis was inhibited by mannosamine. Mannosamine is known to inhibit biosynthesis of the GPI-anchor by preventing assembly of the glycan core (Yuan-tseng et al., 1992). Thus cells were pulsed with the radiochemical analogues described above either in the absence, or presence of varying concentrations of mannosamine. Following this, the raft-localized sIgD was isolated and the extent of associated radioactivity was determined. As shown in Figure 1.19, inclusion of mannosamine resulted in a dose-dependent inhibition in the incorporation of glucosamine, mannose,
and the fatty acid mixture. These results further confirm the presence of a GPI-linked subset of sIgD receptors.

Figure 1.19 Generation of raft-localized sIgD receptors depends on GPI-biosynthesis.

Cells equilibrated with tunicamycin were cultured either in the absence or presence of the indicated concentrations of mannosamine and then pulsed with radiochemical analogues of either glucosamine (GA), mannose (MN), or the combination of fatty acids (FA). The extent of radioactivity incorporated into the de-glycosylated IgD heavy chain of DIS fraction is represented here.

Importantly, mannosamine-treatment of cells also resulted in a near complete, but specific, abrogation of the anti-IgD-dependent cAMP₁ response as shown in Figure 1.20a. The specificity of this effect was readily apparent from the fact that the corresponding responses to either forskolin, or anti-I-A antibodies were unaffected (Fig. 1.20a). Finally, in contrast to the effects on cAMP₁, mannosamine-treatment had no effect on the anti-IgD-dependent mobilization of Ca²⁺ (Fig. 1.20b). This suggested that the cAMP₁, but not the Ca²⁺, response to stimulation of resting B cells with anti-IgD was dependent upon the integrity of the intracellular GPI-biosynthetic pathway.
Figure 1.20 Effect of mannosamine on anti-IgD-dependent cAMP₁ and Ca²⁺ response.

In panel a, either mannosamine-treated (10 mM; groups 1, 3, 5 and 6) or -untreated (groups 2 and 4) were stimulated with either anti-IgD (groups 2 and 3) or, as a control, with anti-I-A (groups 4 and 5). An additional set of mannosamine-treated cells was also stimulated with forskolin (10 μM, group 6). The resultant induction in cAMP₁ levels was then determined. Panel b shows the resultant Ca²⁺ response obtained upon stimulation of either mannosamine-treated (10 mM, profile 1), or -untreated cells (profile 2) with anti-IgD. The profile numbered as three depicts basal levels in mannosamine-treated, but unstimulated cells.

These findings, therefore, confirm the presence of a novel GPI-linked isoform of sIgD receptors on murine resting B cells. This isoform of receptor represents less than 4-5% of total sIgD receptor pool and was constitutively localized in rafts. This subset thus could be depleted from the cell surface by PI-PLC treatment and was also dependent upon the integrity of the GPI biosynthetic pathway. In addition to this, functional role of this receptor subset was also identified. It was shown that upon crosslinking this isoform of IgD specifically mediates cAMP₁ mobilization. The resultant recruitment of cAMP-dependent signaling pathways appears to play a critical role during expression of GC specific markers.
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GPI-linked isoform of IgD mobilizes cAMP.

Results obtained so far reveal the presence of a raft associated GPI-anchored subset of surface IgD. It was also shown that this subset upon crosslinking produced cAMP\(_i\), on the contrary CD79a-CD79b linked receptor specifically resulted in the Ca\(_{\text{2+}}\) mobilization with out affecting the cAMP\(_i\) generation. These observations however led to a hypothesis that the activities of two receptor subsets could be linked. To check for such possibility following experiments were performed. We examined whether the GPI-linked isoform of receptor can function independently or it requires the signaling from the transmembrane form of receptor, for the cAMP\(_i\) induction. For this, genistein which is a broad range protein tyrosine kinase inhibitor and known to block BCR dependent signaling was employed. As mentioned earlier genistein completely inhibited the anti-IgD mediated surface induction of markers. Further, genistein completely blocked the tyrosine phosphorylation upon anti-IgD stimulation. As shown in Figure 1.2a, while inclusion of genistein completely inhibited the mobilization of Ca\(_{\text{2+}}\) it had no detectable effect on anti-IgD dependent cAMP\(_i\) generation (Fig. 1.2b). This, therefore, suggests that the signaling function of the GPI-anchored IgD is independent of that of the transmembrane form of this receptor.
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Figure 1.21 cAMP-dependent signaling by GPI-linked sIgD is independent of the transmembrane sIgD.

Panel a shows the Ca^{2+} response in cells stimulated with anti-IgD either in the presence (profile 2) or absence (profile 1) of 60μg/ml genistein. Panel b represents the cAMP_{1} induction in response to anti-IgD stimulation where various groups indicated are as follows: 1. genistein-untreated cells; 2. genistein-treated cells; 3. cells treated with genistein and PI-PLC; 4. genistein treated cells stimulated with forskolin as a positive control.

To finally prove the origin of cAMP_{1} response, J558L cells were transfected with the construct encoding IgD. As mentioned earlier these cells are deficient for CD79a and, therefore, do not express the CD79a-CD79b associated transmembrane form of receptor. Rather, they have been shown to express the GPI anchored receptor IgD upon transfection (Venkitaraman et al., 1992; Weinands and Reth, 1992). As shown in Figure 1.22a, transfected cells expressed sIgD, which was sensitive to PI-PLC treatment – confirming the expression of a GPI-linked receptor. Further, the surface expression was completely abolished in the presence of mannosamine. These GPI-anchored receptor expressing transfected cells were then analyzed for their ability to induce cAMP_{1}. Stimulation with anti-IgD indeed induced cAMP_{1} response in these transfected cells (fig. 1.22b). This was completely abolished upon treatment with either PI-PLC or mannosamine, (fig. 1.22b). These data therefore demonstrate that the cAMP_{1} generation is an inherent property of GPI-anchored IgD, which is independent of its transmembrane counterpart.
Figure 1.22  GPI-linked isoform of IgD mobilizes cAMP₁ independent of transmembrane receptor.

J558L cells were transfected with a retroviral vector encoding for the heavy chain of IgD. Panel a represents the surface expression of IgD in transfected cells (thick line). Thin line depicts mock transfected cells and dashed line shows transfected cells after treatment with PI-PLC. Transfected cells were also stimulated with anti-IgD and resultant cAMP₁ response was measured. Results are shown in panel b where the groups indicated are as follows: 1, untransfected cells; 2, cells mock transfected with vector only; 3, transfected cells; 4, transfected cells after treatment with PI-PLC; 5, transfected cells after treatment with mannosamine.

Graded responsiveness of resting B cells depending on stimulation thresholds.

Results discussed so far revealed that the sIgD is present in two different isoforms that differ in terms of their mode of expression. These two forms of receptors were also distributed unequally with the GPI-anchored subset constituting less than 5% of the total sIgD pool. This observation also suggests that the extent of crosslinking of receptor may regulate the functional outcome. In other words, depending upon the strength of stimulus, the two isoforms of receptor may influence the extent of activation of preimmune B-cells. To test for such a possibility, resting cells were stimulated with varying concentrations of anti-IgD, and the resultant effects on both capacitative Ca²⁺ influx and cAMP₁ induction was monitored.
Results obtained from such experiments are presented in Figure 1.23a. Peak levels of Ca\(^{2+}\) influx was readily achieved upon stimulation with an anti-IgD dose of as low as 1 \(\mu\)g/ml. Further, the Ca\(^{2+}\) response was unaffected by the PI-PLC treatment where similar profiles were obtained in both PI-PLC treated or untreated cells. This suggested that the anti-IgD dependent Ca\(^{2+}\) mobilization was completely independent of GPI-linked receptor isoform, even in low doses of antigen. Contrary to the Ca\(^{2+}\) response, cAMP\(_{1}\) mobilization could not be observed when low concentrations of anti-IgD were used. Rather, maximal levels of cAMP\(_{1}\) could only be attained when at least 5 \(\mu\)g/ml of anti-IgD was employed (Fig. 1.23a). Further, as expected, this cAMP\(_{1}\) response was completely abolished in PI-PLC treated cells at all the concentrations tested. This differential anti-IgD requirements for the induction of these two pathways is entirely consistent with the observed distribution of the sIgD receptor subsets, wherein the CD79a/CD79b associated form dominates to the extent of greater than ninety-five percent. It is thus proposed that low doses of sIgD would selectively trigger the transmembrane form of sIgD receptor resulting in maximal Ca\(^{2+}\) response. However the triggering of GPI-linked receptor subset, which represents only 4-5% of total receptor, would require higher doses of anti-IgD. Thus, the anti-IgD concentration by influencing the receptor occupancy will determine the quality of signals generated upon stimulation and the subsequent fate of B cells.

The potential physiological consequences, of differential signaling responses to anti-IgD concentrations were also examined. As shown in Figure 1.23b. While induction of activation markers such as I-A and CD86 could readily be achieved by stimulation of cells with doses as low as 1 \(\mu\)g/ml of anti-IgD, peak expression of PNA-R and GL7 was observed only at anti-IgD concentrations of 5 \(\mu\)g/ml (Fig. 1.23b). These results are consistent with our earlier findings in Figure 1.23a, where the BCR-dependent phosphoinositol-signaling pathway was shown to be sufficient for inducing I-A and CD86, whereas for the upregulation of the GC markers cooperative interactions between the cAMP-signaling and phosphoinositol-signaling pathway was required. As shown in figure 1.24a, induction of cAMP\(_{1}\) requires stimulation of cells with a significantly higher concentration of anti-IgD.
Figure 1.23 Stimulation thresholds influence the cellular response.

Cells were stimulated with varying concentrations of anti-IgD and the resultant effects on both Ca²⁺ influx (open circles) and the cAMP₁ response (closed circles) are depicted (Panel a). The corresponding results obtained with PI-PLC treated cells were also shown (Ca²⁺, open triangles; cAMP₁, closed triangles). In panel b, the effects of anti-IgD dose on the cell surface expression of CD86 (open circles), I-A (closed circles), PNA-R (open triangles) and GL7 (closed triangles) are depicted. These results, therefore, support that, by contributing the cAMP signaling pathway, the GPI-anchored subset of sIgD plays a central role in regulating the activation status of resting B cells, in response to an antigenic stimulus. To further probe the level at which such effects might be mediated we investigated the consequence of the differential sIgD triggering on activation of representative BCR-responsive transcription factors. Two transcription factors, NF-kB and the cAMP-response element binding protein (CREB) were selected. The activation of NF-kB can be monitored in terms of its translocation from the cytoplasmic to the nuclear fraction of cell lysates, whereas activation of CREB minimally requires its phosphorylation on the Serine residue at position 133 (Ghosh et al., 1998; Yama-noto et al., 1988). Thus cells, either untreated or treated with PI-PLC, were stimulated with 5 μg/ml of anti-IgD and the consequent effect on both NF-kB and CREB determined by Western blot analyses. As shown in Figure 1.24a, stimulation of untreated cells with anti-IgD resulted in the nuclear translocation of a significant fraction of the p65 subunit (Rel A) of NF-kB. This, however, was markedly inhibited in PI-PLC treated cells (Fig. 1.24a). The specificity of this effect could be demonstrated by the fact
that treatment with PI-PLC had no effect on the anti-IgM-dependent activation of Rel A (Fig. 1.24a). Similarly, PI-PLC treatment was also found to inhibit anti-IgD-dependent phosphorylation of CREB at Ser133 by greater than 70%, but with no effect on that stimulated by anti-IgM (Fig. 1.24b). Thus cooperative contributions from the cAMP-signaling pathway can also detected in the early events that follow stimulation of cells with anti-IgD.

Figure 1.24 Effect of PI-PLC treatment on transcription factor activation.
Nuclear (NE) and cytoplasmic (CE) extracts were prepared from unstimulated cells or cells stimulated either with anti-IgD or anti-IgM and resolved on 10% polyacrylamide gel. Panel A shows the results of a western blot analysis of the nuclear (NE) cytoplasmic(CE) extracts with antibodies specific for the p65 subunit of NF-kB (Rel A). Panel B shows the western blots obtained when the nuclear extract was probed with antibodies specific either for the Ser133 phosphorylated form of CREB (p-CREB), or for the CREB protein (CREB).

Consistent with the observations in Figures 1.24 the relative extent of activation of NF-kB, and Ser133 phosphorylation of CREB, was also dependent upon the dose of anti-IgD employed. Thus, stimulation of cells with 1.0 μg/ml of anti-IgD yielded a ratio of nuclear to cytoplasmic Rel A of 0.15 ± 0.06 (n = 4) following a densitometric analysis of the resultant Western blots (the corresponding value for unstimulated cells was 0.08 ± 0.03). As opposed to this, stimulation of cells with 5.0 μg/ml of anti-IgD resulted in a three-fold enhancement in this ratio, giving a value of 0.48 ± 0.12 (n = 4). This was also
true for CREB phosphorylation at Ser133, where the ratios of phosphorylated CREB to the total CREB protein obtained were 0.57 ± 0.14 and 0.11 ± 0.04 (n = 4) for cells stimulated with 5.0 and 1.0 μg/ml of anti-IgD respectively. The corresponding value for unstimulated cells was 0.01 ± 0.05.

The efficiency of transcription factor activation at high doses of anti-IgD could be further shown to be mediated by cAMP. Thus, the increased activation of both CREB and NF-kB in PI-PLC untreated cells, at an anti-IgD concentration of 10 mg/ml could be demonstrated to be inhibited by H89, an inhibitor of the cAMP-dependent kinase PKA (Fig. 1.25). Further, the absence of a high anti-IgD dose effect seen in PI-PLC treated cells (see Figure 1.25) could also be overcome by supplementing the culture medium with dibutyryl cAMP (db-cAMP), a cell permeable analogue of cAMP (Fig. 1.25) Also important in this context is finding that, in these latter experiments, the effects of db-cAMP was inhibited in the presence of H89 (Fig. 1.25).

![Graph showing the effects of H89 and PI-PLC treatment on anti-IgD dependent activation of transcription factors.](image)

**Figure 1.25** The effects of H89 and PI-PLC treatment on anti-IgD dependent activation of transcription factors.

PI-PLC treated or untreated cells were either left unstimulated or stimulated with 10μg/ml of anti-IgD in the absence or presence of H89. Wherever indicated db-cAMP was used in
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concentration of 100μM. Activation of NF-κB is represented as the ratio of nuclear extract to cytoplasmic extract, and activation of CREB is shown as the ratio phosphorylated to the unphosphorylated.

The cumulative results presented in Figure 1. 23 - 1.25 therefore, highlight the regulatory role played by the GPI-anchored subset of sIgD in defining the activation status of a resting B cell following its encounter with an antigen. Importantly, the data presented here reveals that the effects of GPI-sIgD are principally mediated by the cAMP-signaling pathways that it recruits which, in turn, contributes by regulating the intracellular BCR-dependent signaling thresholds involved in upregulation of the activation versus the GC markers. Finally, our observation that GPI-linked sIgD constitutes the minority subset (<5%) of the total sIgD pool also reveals how antigen–dependent regulatory influences may be exerted.

The GPI-linked sIgD receptor subset is necessary for optimizing GC responses from primary B cells.

Although our cumulative data so far provided support for the functional relevance of the GPI-anchored sIgD, it was necessary to directly determine the biological significance of this receptor subset. For this PI-PLC treated were stimulated, either in the presence or absence of 100μM db-cAMP, with 5.0μg/ml of anti-IgD. Following this, cells were either analyzed for the resulting phenotypic modifications, or, processed for an evaluation of their GC seeding potential. As shown in figure 1.26a, with the exception of CD86, PI-PLC treatment led to a varied but significant inhibition in upregulation of all the markers studied. This inhibition, however, could be overcome by the inclusion of db-cAMP during the stimulation period (Fig. 1.26a).

Consistent with effects on marker upregulation, treatment of resting B cells with PI-PLC also resulted in an attenuated GC response, following stimulation with anti-IgD and subsequent adoptive transfer (Fig. 1.26b). These results, therefore, validate the
functional significance of the GPI-linked sIgD receptors, and the cAMP-signaling pathway that they recruit.

Figure 1.26. PI-PLC treatment of resting B cells inhibits both phenotypic and GC responses.

In panel a, PI-PLC treated cells were stimulated with anti-IgD either in the absence (hatched bars) or presence (crossed bars) of db-cAMP. The upregulation of various markers is shown. For comparison, the results obtained with untreated cells are also shown (open bars). Magnitudes of the GC responses of the experimental groups in a are shown in panel b.
The population of B lymphocytes present in peripheral lymphoid tissues is dynamically maintained by processes that balance between the continuous output from the bone marrow, and proliferation in response to antigens with terminal differentiation and death. The choices between the alternate fates of proliferation, differentiation and death are largely determined by intracellular signaling cascades triggered by receptor-ligand interactions. The BCR is the central regulator of B-cell fate. Intracellular signaling through the BCR is required for survival, as well as for antigen specific immune response. At least four types of extracellular inputs govern the response to an antigen. These are- the concentration of antigen, the avidity with which an antigen interacts with B cell, the timing and duration of antigen encounter, and the association of B cells with other lymphocytes. Although a multitude of signal transduction events are known to be activated upon BCR ligation the critical parameters that determine the biological outcome of the signal transduction cascades, however, are not yet understood completely.

One important level at which regulation occurs is during the induction of a primary humoral response. Here the affinity for antigen as well as the degree of receptor occupancy play important roles in defining both the quality of intracellular signals generated, and the potential of activated B cell clonotypes to seed GCs (Agarwal et al., 1998; Glynne et al., 2000; Cambier and Ransom, 1997; Kouskoff et al., 1998). The present study was undertaken to identify at least some of the underlying mechanisms by which affinity of BCR for antigen may regulate the functional status of preimmune B cells. To address this question, we first developed an experimental system where resting B cells were purified from the BALB/c IgH² mice and treated with F(ab)² fragment of anti-IgD. Following stimulation, B cells attain two distinct phenotypic states of activation. They either selectively express markers characteristic of an activation phenotype (i.e. sIgD^o, I-A^hi, CD86^hi), or, in addition to these markers, they also display increased expression of markers typical of the GC phenotypic state (i.e. PNA-R^hi, CD24^hi, GL7^hi) (Fig. 1.1). The differential attainment of the two phenotypic states was also functionally relevant as these cells could form GCs only upon the expression of the GC related markers as ascertained by our modified adoptive transfer protocol. In this protocol anti-IgD stimulated B cells from BALB/c IgH² mice were loaded with T-cell
epitope CT3 and subsequently transferred into the CT3 primed BALB/c IgH<sup>+</sup> mice. Stimulation with optimal concentration of anti-IgD yielded target cells with the potential to seed GC, although the latter process was strictly dependent upon the availability of T cell help (Fig. 1.2).

Thus, these phenotypic changes induced upon stimulation of resting B cells with anti-IgD provided us a good model system to explore how regulatory controls are variably enforced. Ideally, a clear understanding of such a process would first require a thorough knowledge of the entire intracellular biochemical machinery that is mobilized upon BCR cross-linking. This, however, constitutes a monumental task at the present time, which is further hampered by the complex networking of signaling cascades that is known to occur, and also our current ignorance of the spectrum of target genes that they influence. It was for this reason that we adopted a more restricted strategy that first focused on identifying some of the key intermediates involved, and then monitored for their effects.

In the course of this study cells were stimulated in the presence of various pharmacological inhibitors. By using EGTA, which is a known inhibitor of Ca<sup>2+</sup> influx, PKC inhibitor- Calphostin C and H-89 - a PKA inhibitor, we have identified three second messengers. These are - Ca<sup>2+</sup>, PKC and cAMP. Our results suggest that while Ca<sup>2+</sup> and PKC play obligatory roles in the surface expression of both the activation and GC markers, the effect of cAMP was restricted to GC markers (Fig. 1.8). These second messengers are recruited at suboptimal levels following BCR crosslinking. We have also shown that a crosstalk between these suboptimally recruited BCR –dependent second messengers is critical for regulating between the phenotypically distinct states of activation. As shown in Figure 1.13 while activation of the phosphoinositol pathway was alone sufficient to confer the activated phenotype, acquisition of both the GC phenotype and the GC seeding capability required the additional recruitment of cAMP-dependent pathways. Thus the nature and extent of this crosstalk defines the spectrum of activation markers that are expressed and, therefore, the biological outcome.

An important aspect of this study is the identification of GPI-anchored isoform of receptor. This receptor isoform constitutes a minority fraction of the total slgD pool, and is constitutively localized within rafts microdomains of the plasma membrane (Fig. 1.17).
An important distinction between this subset and the transmembrane pool of sIgD was its ability to mobilize cAMP₁ - but not Ca²⁺ - dependent signaling pathways. In other words these two receptor isoforms also represent a segregation of sIgD signaling function, which provided a platform for regulating activation thresholds via crosstalk between signals generated from the two sIgD isoforms. Thus, cAMP dependent pathways, induced by GPI- linked sIgD stimulation, act in concert with signals generated from the transmembrane sIgD to further optimize GC responses from activated primary B cells (Fig. 1.14). This is probably mediated by augmenting activation of at least some of the BCR responsive transcriptional activators, and the subsequent upregulation of GC markers (Fig. 1.24). Nevertheless, the fact that this receptor isoform plays an important role in optimizing GC responses clearly point to its physiological relevance (Fig. 1.26).

Although stimulation of B cells with anti-IgM does not induce a cAMP₁ response, previously it has been demonstrated that signaling through sIgM is also sensitive to cAMP₁ dependent signaling pathways (Natarajan et al., 2001). Thus, simultaneous ligation of sIgM and CD54 on B cells led to an increase both in CREB phosphorylation, and the phenotypic response. This augmentation was mediated by cooperative interactions between the sIgM – dependent phosphoinositol signaling, and the CD54 –activated cAMP₁ -dependent pathways (Natarajan et al., 2001). It is, therefore, possible that by mobilizing cAMP₁ the GPI-anchored sIgD may also function to enhance sIgM dependent signaling in resting B cells.

While both sIgM and sIgD are thought to be functionally equivalent in resting B cell, it is generally accepted that it is the sIgD class of receptor that serves as the principal sensor for antigen. This is because of the increased expression of this isotype over IgM, and also the fact that sIgM is more rapidly downregulated by antigen than is sIgD (Brink et al., 1995). In fact it has been suggested that the sIgD receptors play a critical role in the humoral defense against pathogens undergoing rapid expansion and mutational drift upon entry into the host (Roes and Rajewsky, 1993). Such an inference was based upon the fact that IgD –knockout mice displayed delayed antibody production and affinity maturation in response to model T- dependent antigens (Roes and Rajewsky, 1993). Similarly, we have also observed that the number of GCs obtained from anti-IgM...
stimulated resting B cells was significantly reduced in comparison with that obtained from anti-IgD stimulated cells.

The transmembrane and GPI-linked isoforms of sIgD are distributed unequally, with the former making up more than 95% of the total sIgD pool. This unequal distribution probably permits graded responsiveness of primary B cells, depending upon the strength of the antigenic stimulus (Fig. 1.23). Thus high antigen concentrations or affinity would ensure saturating receptor occupancy so that both subsets are stimulated, and an optimal GC response is induced. Conditions that limit receptor occupancy (i.e. where antigen concentration or affinity is low) would selectively stimulate the dominant transmembrane subset, leading to B cells expressing only an activated phenotype, and with an attenuated GC seeding capacity. Thus it is the simple arithmetic of receptor partitioning, which integrates into distinct signaling thresholds, that contributes towards the acumen of preimmune B cells.

In summary we demonstrate, in murine resting B cells, that minority fraction of the sIgD receptors exists in a form that is GPI-linked. This isoform is constitutively localized within raft microdomains of the cell membrane, and is distinguished from its transmembrane counterpart by its ability to activate cAMP\(\text{I}^1\) dependent signaling pathways. The cAMP\(\text{I}^1\) signaling pathways activated through the GPI-linked sIgD contribute through cooperative interaction with intracellular signals generated from the transmembrane sIgD. This, in conjunction with the unequal distribution of these two sIgD isoforms, provides a mechanism by which the target preimmune B cells can be differentially activated depending upon the strength of antigenic stimulus experienced.
In the previous chapter we have shown that stimulation of murine resting B cells with anti-IgD resulted in the upregulation of various cell surface markers, in addition to the downregulation of sIgD. Some of these markers were typical of activated B cells, whereas others characterized the GC phenotype. These phenotypic changes induced upon IgD triggering were also shown to be functionally relevant by demonstrating that these activated B cells were fully capable of forming GCs upon adoptive transfer into naive recipients. Further, upon stimulation with anti-IgD corresponding transcripts levels were also found to increased suggesting that the surface densities of these molecules probably derived from the observed increase in their corresponding mRNAs.

As discussed earlier, although a number of B cells are activated following encounter with an antigen, the GC seeding capacity is restricted to only the high affinity subsets. This observation suggested that BCR dependent events might play a regulatory role in defining the functional and phenotypic properties of activated B cells. Some insight into how this might have achieved was provided in the previous chapter, where we have identified and characterized the existence of minority subset of GPI-linked sIgD receptor. Further, the unequal distribution of the transmembrane versus the GPI-linked form also provided a clue as how variations in the nature of Ag–BCR interaction may regulate the functional status of primary B cells. To study the physiological consequences of such regulations we, in the present chapter, explore how variations in the Ag-BCR interactions in fact influence primary B cell responsiveness.

Properties characteristic of activated and GC B cells are acquired at distinct rates.

As shown in earlier chapter, stimulation of B cells with anti-IgD resulted in the increased cell surface expression of activation and GC associated markers (Fig 1.1). We determined the time required for induction of individual marker. For this, B cells from BALB/c IgH\textsuperscript{a} mice were stimulated with anti-IgD and aliquots were removed at various time points for an examination of cell surface expression of activation and GC markers. As shown in figure 2.1, the maximal effects on surface IgD downregulation and CD86 upregulation were obtained by 12 hrs of stimulation. On the other hand, peak levels of
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CD24 and B7.1 were induced only after 60 hrs of culture with anti-IgD (Fig. 2.1). Surface induction of MHC II was achieved by 24 hrs, however, maximal surface expression of both PNA-R and GL7 required 48 hrs of stimulation. Thus, the activated phenotype of B cells (i.e. I-A<sup>hi</sup>, CD86<sup>hi</sup>) was achieved early, within 24 hrs of antigen trigger. However, the acquisition of GC phenotype (i.e. PNA-R<sup>hi</sup>, CD24<sup>hi</sup>, GL7<sup>hi</sup>) could only be obtained after an extended stimulation period of at least 60 hrs.

![Figure 2.1 Kinetics of induction of the activated and the GC phenotype in anti-IgD stimulated B cells.](image)

The functional relevance of the differential kinetics of induction for the activation and GC markers was also ascertained. For this, resting B cells from BALB/c IgH<sup>8</sup> mice were stimulated with anti-IgD for the times indicated in figure 2.1, and their ability to populate GCs was subsequently analyzed (material and methods). At day 8 after transfer, spleens were removed and sections were stained for PNA-R positive and IgH<sup>8</sup> allele specific cells. Results from such experiments revealed that an efficient GC response was obtained only when cells were stimulated for at least 60 hrs. The number of GCs
obtained was reduced by at least 50% when cells that were stimulated for only 48 hrs were transferred (Fig. 2.2). Stimulation periods of less than 48 hrs resulted in a further loss in GC forming ability, with the number reaching barely detectable levels for cells treated with anti-IgD for 24 hr (Fig. 2.2). These findings therefore suggest that the temporal distinctions do exist between the acquisition of an activated versus a GC phenotype, with the former proceeding the latter.

![Graph showing kinetics of induction of GC forming ability in anti-IgD stimulated B cells.](image)

**Figure 2.2** Kinetics of induction of GC forming ability in anti-IgD stimulated B cells.

*Cells were stimulated with a 10 μg/ml final concentration of anti-IgD. Following this, aliquots were removed at the indicated times and the cells were transferred in the CT3 primed mice for the evaluation of their GC forming ability. The average number of GCs obtained, in the spleen sections of recipients, are shown here.*

**Duration of BCR triggering modulates the activation status of resting B cells.**

The differences in stimulation requirement for induction of the activation and GC markers observed suggested that the variations in the duration of BCR triggering might also regulate the phenotypic or functional status of B cells. To test for this, cells were stimulated with anti-IgD for various times. Following this, the stimulant was removed and cultures were continued for the total period of 60 hrs prior to analysis. This was done
in order to normalize for the possible differences in the time needed for regulated transcription and translation of the individual molecules. As shown in Figure 2.3, the minimum time of BCR triggering required for induction of activation and GC markers differed considerably. A stimulation time of 10 minutes was sufficient to yield maximal effects on both downregulation of slgD, as well as on the CD86 induction (Fig. 2.3). Peak surface densities of MHC II were observed when the receptor was triggered for 30 min, whereas a stimulation period of 4-6 hr was required for the optimal induction of both GL7 and PNA-R (Fig. 2.3). Finally, saturable surface induction of CD24 and CD80 was observed only upon sustained stimulation with anti-IgD for at least 30 hrs. (Fig. 2.3)

These results, therefore, reveal the existence of distinct BCR triggering thresholds for induction of both activation related, and, GC markers. A shorter duration of antigen-receptor interaction was capable of inducing the activated phenotype (i.e. CD86<sup>hi</sup>, I-A<sup>hi</sup>). However, the GC phenotype could be obtained only upon sustained triggering of receptor that continued for at least 30 hours.

**Figure 2.3** The expression of the activated versus the GC phenotype requires distinct slgD triggering times.

*Cells were stimulated with anti-IgD with final concentration of 10 μg/ml for the indicated times. Following this the stimulus was removed by extensive washing, and the culture continued such that the total culture period for all groups was 60 hours. The resultant fold induction of the individual markers obtained is represented as a function of stimulation time. Standard error was 5-10% of the mean.*
The requirement of differential triggering times of BCR, for induction of the various phenotypic markers, was also correlated with the acquisition of GC seeding competency by these B cells. For this, resting B cells were purified from BALB/c IgH^m mice and stimulated with anti-IgD for various times as described above. These cells were then processed and evaluated for their ability to form GCs as described earlier. The results from such experiments are shown in figure 2.4. As expected, maximum numbers of germinal centers were obtained in the spleen sections where cells were triggered for 30 hr or more. As seen earlier, this also represented the minimal period necessary to induce the markers associated with the GC phenotype (Fig. 2.4). The number of GCs reduced dramatically by 80% when cells were stimulated for 30 min -1 hr were used, whereas only 50% GCs were observed when duration of receptor triggering employed was between 4- 6 hrs (Fig. 2.4).

![Figure 2.4](image)

Figure 2.4 The effect of distinct sIgD triggering times on the GC seeding potential of anti-IgD stimulated cells.

Resting cells were stimulated with anti-IgD with final concentration of 10 µg/ml for the indicated times as described in results. Following this, cells were evaluated for their ability to form GCs in CT3-primed BALB/c IgH^p mice. The number of GCs obtained from spleen sections of recipients are shown as a function of stimulation time.
Thus B cells bearing an activated phenotype (sIgD$^{lo}$, MHCII$^{hi}$, CD86$^{hi}$) could be obtained with relatively shorter ligation period of the receptor. These cells, however, were incapable of seeding GCs. The optimal acquisition of GC seeding capacity necessitated prolonged BCR triggering over a period of 30 hrs (Fig. 2.4), a time frame consistent with that required for induction of the GC phenotype (Fig. 2.3). These results therefore suggest that the duration of sIgD crosslinking constitutes at least one regulatory parameter that defines the activation status of resting B cells. Further they also reveal that generation of the activated versus the GC phenotype involves distinct BCR dependent thresholds.

The concentration of available antigen also dictates the activation status of the resting B cells.

As shown in figure 2.3, the generation of the activated versus the GC phenotype of a primary B cell differs in their requirement of time for receptor stimulation. These results, however, also raised the possibility that the modulation of activation of resting B cells may also be dependent upon the concentration of the available antigen. To test for this, cells were stimulated with varying concentrations of anti-IgD and the resultant effects on cell surface markers expression were monitored. These results are shown in Figure 2.5 where a dose dependent response to anti-IgD can clearly be observed. For instance, CD86 induction could be maximally observed upon stimulation with anti-IgD at concentrations as low as 0.5μg/ml (Fig. 2.5). As opposed to this, induction of peak surface densities of I-A, PNA-R and GL7 required stimulation with at least 2 μg/ml of anti-IgD (Fig 2.5). Finally, optimal upregulation of CD80 and CD24 was observed only upon stimulation of cells with 5 μg/ml or more of anti-IgD (Fig 2.5).
Figure 2.5 Stimulation thresholds for the acquisition of the activated and the GC phenotype.

Cells were cultured in the presence of the indicated concentrations of anti-IgD for a total period of 60 hours and processed for an analysis of the cell surface marker expression. The modal fluorescent intensities (MFI) for PNA-R (a), I-A (b), CD86 (c), CD24 (d), GL7 (e), and CD80 (f) are depicted here.
In the next set of experiments, the above effects were sought to be correlated with the GC seeding ability by these cells. As shown in Figure 2.6, peak GC responses were obtained only from those cells that had been stimulated with anti-IgD concentrations of at least 5 μg/ml. Stimulation with decreasing concentrations of anti-IgD resulted in a concomitant decrease in the magnitude of the GC response, being reduced by greater than 80% when cells stimulated with 1 μg/ml of anti-IgD were employed (Fig. 2.6). Thus in addition to the BCR triggering thresholds, concentration of available antigen also appears to regulate the extent of activation status of preimmune B cell.

![Graph showing the effect of anti-IgD concentration on GCs per ten sections](image)

**Figure 2.6** Effect of anti-IgD concentration on the functional status of resting B cells.

*Cells were cultured in the presence of the indicated concentrations of anti-IgD for a total period of sixty hours. Following this, cells were adoptively transferred as discussed in methods. The average number of GCs obtained are plotted against the anti-IgD concentrations.*

The above results again reveal a direct correlation between the acquisition of the GC phenotype by the B cells, and their ability to populate GCs. Importantly, these data also characterize that antigen concentration - dependent threshold exist that define the extent to which the target preimmune B cell is activated.
Germinal center formation is dependent on second messengers.

Crosslinking of the BCR results in the eventual recruitment of at least four major intracellular pathways (Campbell, 1999; Defranco, 1997). These include those dependent upon the activation of PLC\(_\gamma\), the rho family and ras family of GTPases, and PI3Kinases. PLC\(_\gamma\) activation leads to the hydrolysis of phosphoinositide PIP2 which, in turn, results in the generation of two second messenger molecules – inositol triphosphate (IP3) and diacyl glycerol (DAG). IP3 diffuses in the cell to engage IP3 receptors on the ER and, thereby, release Ca\(^{2+}\) from internal stores. This is then followed by Ca\(^{2+}\) influx through calcium release activated (CRAC) channels. On the other hand, DAG in conjunction with cytosolic Ca\(^{2+}\) leads to the activation of certain isoforms of PKC (Beschop and Cambier, 1999).

The downstream mediators of the PLC\(_\gamma\) pathway, Ca\(^{2+}\) and PKC, are known to play important roles in the upregulation of markers that characterize both the activated and GC phenotype of the B cells (discussed in Results - 1). With respect to Ca\(^{2+}\), our own results suggest (see Results I) that it is the capacitative influx phase that is the most important.

In order to identify the possible involvement of Ca\(^{2+}\) in an anti-IgD dependent GC response, resting B cells from BALB/c IgH\(^a\) were treated with anti-IgD either in the presence or absence of either EGTA or TMB-8. Following this cells were transferred into peptide CT3 primed BALB/c IgH\(^b\) recipients. Eight days later spleens were removed and sections were stained for IgH\(^a\) allele specific germinal centers. As shown in figure 2.7, the GC response was completely inhibited in the presence of either EGTA or TMB-8. These results implicate that induction of an anti-IgD dependent Ca\(^{2+}\) response represents an obligatory step during the acquisition of GC seeding potential by these primary B cells. Mobilization of calcium from the extracellular medium can also be achieved artificially by using ionomycin, a calcium ionophore, which leads to an increase in the cytosolic Ca\(^{2+}\) levels (Dolmetsch et al. 1998). In the previous chapter, we have already demonstrated that, treatment of B cells with ionomycin results in the induction of both activation and GC related markers. In keeping with these results, ionomycin treatment was also found to confer the GC seeding potential to the resting B cells.
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(Fig. 2.7). The number of germinal centers obtained in this case was comparable to that obtained with anti-IgD stimulated cells. The effect of ionomycin was completely blocked by the simultaneous addition of EGTA, whereas Calphostin C (PKC inhibitor) had no inhibitory effect indicating the specificity of Ca\textsuperscript{2+} dependent response.

We also investigated the involvement of PKC in conferring GC forming ability during anti-IgD stimulation of B cells. For this cells were preincubated with calphostin C, a specific inhibitor of protein kinase C, and triggered with anti-IgD. As shown in figure 2.7, between 80-85 GCs per ten sections were obtained from cells that were stimulated in the absence Cal C. This number was reduced by as much as 80-90% for the corresponding cells that were stimulated in the presence of Cal C. This result, therefore, implicates the importance of PKC during an anti-IgD driven germinal center response. Similar results were also obtained in the presence of another PKC inhibitor, staurosporine. To further confirm the role of PKC during the phenotypic and functional activation of B cells, phorbol esters, which are known to activate protein kinase C, were used. The effect of phorbol esters on germinal center formation was analyzed by incubating the cells with saturating concentration of phorbol myristate acetate (PMA), and then transferring these cells into IgH\textsuperscript{b} mice. Fully formed GCs were observed in such spleens and numbers were comparable to that obtained from a parallel set of cells that were stimulated with anti IgD. These collective results thus implicate an obligatory role for Ca\textsuperscript{2+} and at least some of the conventional PKC isoforms in BCR mediated surface expression of markers and the ability of these target cells to form germinal centers.
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Figure 2.7. Germinal center formation is dependent upon second messengers. Purified B cells were stimulated with anti-IgD either in the presence or absence of TMB-8, EGTA, or Cal C and processed for their evaluation of GC seeding competency. In parallel, cells were also stimulated with either Ionomycin or PMA. Number of GCs obtained per ten sections is shown here.

Thresholds of second messengers regulate the phenotypic and functional status of resting B cells.

As shown earlier surface expression of both activation and GC markers and GC seeding competency could be induced by artificially activating B cells with Ionomycin and PMA. It was thus of interest to explore the possibility that the threshold barriers which distinguish between the generation of the activated versus the GC phenotype also exist at the level of second messenger recruitment. To probe for this, varying concentrations of either ionomycin or PMA were employed and the effects on both the cell surface marker upregulation as well as the ability of these cells to form GCs were monitored. As discussed in Results I, increasing ionomycin concentrations in the culture medium translated into increased cytosolic Ca$^{2+}$ concentrations as determined by labeling cells with the Ca$^{2+}$ binding dye FLUO-3 AM and analysis by FACS. Similarly treatment of B cells with increasing PMA concentrations resulted in a corresponding,
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dose dependent increase in the PKC activation when measured in terms of the extent of its translocation from the cytoplasmic to the membrane fraction. As shown in Figure 2.8 dose dependent effects of both ionomycin and PMA are clearly evident where increasing concentrations led to an increased efficiency in the induction of the spectrum of cell surface markers studied, as well as in the GC forming ability. Importantly, however, distinct induction thresholds for the individual cell surface markers are again evident (Fig. 2.8). Thus, while maximal induction of both CD86 and I-A could be achieved with either 0.2 nM PMA or 1μM ionomycin, that for the induction of PNA-R and GL7 at least 2 nM PMA or 4 μM ionomycin was needed. Finally maximal increase in surface levels of CD80 and CD24 could only occurred in the presence of 10 nM PMA or 6 μM ionomycin (Fig. 2.8). Thus, while PMA or Ionomycin doses of as low as 0.2 nM or 1μM was sufficient to induce the activated phenotype, the GC phenotype could only be obtained with relatively higher concentrations of PMA or ionomycin.

The observed differences in intracellular messenger requirements for the generation of the two distinct activation states noted above could also be further confirmed at the level of the functional properties. Thus, cells that were treated with varying concentration of either PMA or ionomycin were also analyzed for their GC seeding competency. Optimal GC responses were obtained only from those cells that had been stimulated with 10 nM PMA or 6 μM ionomycin. The number of GCs were comparable to that obtained from a parallel set of cells that were stimulated with anti-IgD. The number of these GCs were reduced by more than 40% when cells stimulated with 2 nM PMA or 4 μM ionomycin were employed instead. The number of GCs were reduced to 20% for cells treated with 1nM PMA or 2 μM ionomycin, with nearly no GC being detected when 0.2 nM PMA or 0.5 μM ionomycin treated cells were transferred (Fig. 2.8). Thus, while the activated phenotype could be obtained with lower concentrations of PMA (0.2 nM) or ionomycin (1 μM), induction of GC phenotype as well as the GC seeding potential required relatively higher concentrations of PMA (10nM) or ionomycin (6 μM). These results, therefore, confirm that distinct Ca$^{2+}$ and PKC threshold exist for the generation of either activated phenotype only (i.e. CD86$^{hi}$, I-A$^{hi}$), or those that also posses the ability to form GCs (PNA-R$^{hi}$, GL7$^{hi}$ & J11D$^{hi}$).
Figure 2.8 Second messengers thresholds regulate the phenotypic and functional status of resting B cells

Resting B cells were either left untreated or treated with varying concentrations of either ionomycin (panel a and c) or PMA (panel band d). Panel a and b depict the resultant induction
suboptimal. Representative results at these two concentrations are shown in figure 2.9. As is evident, B cells were most responsive to anti-I-A, where, stimulation with an antibody concentration of 10 μg/ml resulted in upregulation of all the GC markers studied, but with no effect on CD80 levels (Fig. 2.9 a). Importantly, these cells were also found to be capable of forming GCs upon subsequent adoptive transfer. However the magnitude of both the GC and phenotypic responses to anti-I-A (10 μg/ml) were markedly lower than that obtained upon stimulation of cells with 10 μg/ml of anti-IgD (Fig. 2.9). As opposed to the wider ranging effect of anti-I-A, stimulation with high doses of either anti-CD54 or anti-CD40 led to a selective response where only PNA-R and CD24, but not GL7 and CD80, were moderately upregulated (Fig 2.9). Further, B cells stimulated with either of these latter reagents were also incapable of forming GCs. (Fig. 2.9)
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of the individual cell surface markers. The GC response obtained from these cells is shown in panel c and d.

**Auxiliary signals contribute towards modulation of the activation threshold barrier.**

The results so far reveal the existence of distinct activation thresholds for the antigen dependent acquisition of either an activated or a GC phenotype by resting B cells. This distinction in activation thresholds exists at various levels. The acquisition of an activated or the GC phenotype from an antigen activated primary B cells require distinct duration of receptor triggering and also follow distinct time kinetics for induction. Further they also differ in their requirement for concentration of antigen. Importantly this distinction was clearly visible at the level of the intracellular mediators also. The formation of GCs is, however, a T-dependent process where activation of B cells also involves, in addition to BCR-antigen interaction, signals generated from cognate interactions with antigen specific T cells. The latter includes the interactions between the MHC-peptide complex with the TCR, and those between the co-stimulatory molecules and their receptors, as well as interactions between the adhesion molecules present on both cell types (Clark and Ledbetter, 1994). Intracellular signals generated from each of these interactions, are also known to play a role in modulating functional properties of antigen activated B cells (Bishop and Hostager, 2001). It was, therefore, of interest to examine how contributions from such auxiliary signals could further regulate antigen dependent activation of resting B cells and define their phenotypic status.

Although recruitment of T cell help involves multiple interactions between the B and T cells for the ease of analysis, however, only three B cell surface molecules were selected as the representative targets. These were I-A, CD40, and CD54. For the initial studies, purified resting B cells were individually stimulated with antibodies specific for each of these molecules and the resultant effects on expression of the GC markers and CD80 were monitored. Results from such experiments employing varying doses identified that a concentration of 10 μg/ml produced maximal effects for each of these three antibodies, whereas, an antibody concentration of 0.5 μg/ml proved to be
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Figure 2.9 Individual T cell-derived auxiliary signals have marginal effects on the B cell response.

Resting B cells were either left untreated (cells), or stimulated with either 10 μg/ml (h) or 0.5 μg/ml (l) of either anti-I-A (I-A), anti-CD54 (CD54), or anti-CD40 (CD40). For comparative purposes, a parallel set of cells was also stimulated with anti-IgD. Panel a depicts the resultant induction of the individual cell surface markers. The GC response obtained from these cells is shown in panel b.

After establishing the relative efficiencies of each of the three antibodies described above, we next looked for possible cooperative effects from I-A, CD54 and CD40 towards BCR mediated functional modulation of B cell responses. For these experiments the suboptimal dose (0.5 μg/ml) for each of these antibodies was employed. Resting B cells were triggered with anti-IgD, followed by the addition of either anti-I-A, anti-CD40, or anti-CD54 after 12 hrs of stimulation (Materials & Methods). After 60 hrs of stimulation with anti-IgD, cells were analyzed either for the surface expression of GC markers, or, for their ability to seed GCs upon adoptive transfer. As shown in Figure 2.10, varying but moderate degrees of cooperativity could be observed depending upon the antibody combinations employed. Cells treated with the combination of anti-IgD and anti-I-A showed a significant enhancement in the levels of PNA-R, CD24 and GL7. Combination of anti-IgD and anti-CD40 exerted similar effects on PNA-R, and CD24 however GL7 levels remain unchanged. Addition of anti-ICAM-1 was least effective, resulting only in CD24 upregulation. None of these combinations were however capable of inducing surface CD80 levels. An aliquote of cells from each of these groups was also
transferred into CT3 primed mice for an evaluation of their GC seeding potential. With the exception of cells treated with anti-IgD followed by anti-I-A, where a moderate response was observed none of these combinations employed were capable of generating B cells with GC forming ability (Fig. 2.10).

![Figure 2.10 Cooperative effects of T-cell derived signals towards BCR mediated phenotypic and functional modulation of B cell responses.](image)

**Figure 2.10** Cooperative effects of T-cell derived signals towards BCR mediated phenotypic and functional modulation of B cell responses.

*Cells were stimulated with 0.5 µg/ml of anti-IgD, following which 0.5 µg/ml of either anti-I-A, anti-CD40, or anti-CD54 was added 12 hours later. After a total culture period of 60 hours,*
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cells were processed either for phenotypic modifications (panel a), or for their ability to form GCs (panel b).

The observation in figure 2.10, that at least some degree of cooperativity exists between anti-IgD and the secondary stimuli examined, led us to extend this line of investigation by exploring the effects of additional combination of these reagents. The various antibody combinations tested and their effects on phenotypic modification and GC forming ability are shown in figure 2.11. It is evident from the figure that the tested combinations of any two or three reagents proved to be relatively ineffective, yielding only marginal to insignificant effects on both the cell surface phenotype, as well as on the GC seeding potential (Fig. 2.11).

The only exception to this was the combination of anti-CD40, anti-I-A, and anti-54, which resulted in marginal increase in surface densities of PNA-R, CD24, GL7 and CD80 (Fig. 2.11a). Further, upon subsequent transfer these cells gave a moderate but significant GC response where 30-35 GCs per 10 sections could be detected (Fig. 2.11b). Of the various experimental groups examined, however, it was the combination of the all four antibodies- i.e. anti-IgD, anti-CD40, anti-I-A, and anti-54, that proved to be the most effective. Both the phenotype and GC responses were maximal from this group (Fig. 2.11) and, importantly, were comparable in magnitude to that obtained upon stimulation of cells with 10 µg/ml of anti-IgD (Compare figs. 2.9 and 2.11).
Figure 2.11 The effects of limiting anti-IgD concentrations can be potentiated only by multiple T cell-derived secondary signals.

Cells were left either untreated (cells), or were treated with a suboptimal concentration (0.5 μg/ml) of anti-IgD for twelve hours. At this point, 0.5 μg/ml each of anti-I-A (I-A), anti-CD54 (CD54), or anti-CD40 (CD40) was added in the various combinations indicated. The consequent increase in surface densities in either PNA-R, CD24, GL7, or CD80 in cells from the individual
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groups is shown in panel a. This increase is depicted as fold increase in MFI, in B220\(^+\) gated cells, over that obtained in cells where the secondary stimuli were not added. Shown in panel b are the GC responses obtained from each of these groups, after the cells were loaded with peptide CT3 and transferred into CT3-primed BALB/c IgH\(^\delta\) mice.

Thus cooperative contributions from secondary stimuli appear to be capable of overcoming the activation threshold barrier imposed by BCR dependent pathways. It is important to note, however, that no single secondary stimulus was alone capable of achieving this. Rather, it required the combined effects of anti-CD40, anti-I-A, and anti-54 to maximize responses from cells stimulated with 0.5 \(\mu\)g/ml of anti-IgD. In other words contribution from T cells, at least towards driving antigen dependent activation of B cells, is likely to represent the cumulative effect of multiple interactions established at the cell - cell interface.